

## Measurement of cholic acid synthesis and secretion by isolated rat hepatocytes

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**Summary** Liver cells isolated from normal and cholestyramine-treated rats were incubated as cell suspensions for up to 4 hr in a simple, defined medium. The bile acid concentration in cells plus cell medium was determined by gas-liquid chromatography. Normal hepatocytes synthesized cholic acid at an initial rate of 0.25 nmol/mg cell protein per hr, which is comparable to rates reported from in vivo methods. This rate was increased more than 4-fold when rats were fed a cholestyramine-containing diet for 7 days prior to liver cell isolation. Although cholic acid was secreted into the cell medium during the incubation, it could not be assayed reliably by the hydroxysteroid dehydrogenase assay method, contrary to the reports of Anwer et al. 1975. *Biochem. Biophys. Res. Commun.* **64**: 603 and Gardner and Chenouda 1978. *J. Lipid Res.* **19**: 985.—**Whiting, M. J., and A. M. Edwards.** Measurement of cholic acid synthesis and secretion by isolated rat hepatocytes. *J. Lipid Res.* 1979. **20**: 914–918.

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Bile acid synthesis by the liver balances fecal losses to maintain a constant bile acid pool size within the enterohepatic circulation. The normal low level of hepatic synthesis is under feedback control and, fol-

lowing interruption of the enterohepatic cycle, synthesis is increased several fold by increasing the activity of the rate-limiting enzyme of bile acid biosynthesis, cholesterol 7 $\alpha$ -hydroxylase (1).

Measurement of hepatic bile acid synthesis has been carried out in man and intact animals by isotope dilution, fecal output, conversion of [<sup>14</sup>C]cholesterol to <sup>14</sup>CO<sub>2</sub> (2–5), and also by pool drainage techniques involving a bile fistula (3, 5). However, these in vivo methods are indirect and usually involve collecting samples over several days. Since isolated hepatocytes have been used successfully to study a wide range of metabolic and synthetic activities of the liver at the cellular level, we have measured the rate of bile acid synthesis and secretion in rat liver cells incubated in a simple, defined medium for short periods.

## MATERIALS AND METHODS

### Animals

Male hooded Wistar rats weighing 180–260 g were used for all experiments. Prior to liver perfusion, the rats were fed either rat chow ad libitum or chow containing 4% (w/w) cholestyramine for 7 days. Food intake was unchanged by this diet and was 20 g/day per rat. Bile was collected for 2 min after anesthetizing the rats and cannulating the common bile duct.

### Preparation and incubation of isolated hepatocytes

Hepatocytes were isolated by the following modification of the method of Berry and Friend (6). Rat livers cannulated via the portal vein were perfused (30 ml/min) in situ with Swim's S77 medium con-

Abbreviations: LHS medium, a modified L-15 medium with added butter, albumin and amino acids (7).

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taining 0.1% (w/w) bovine serum albumin fraction V; heparin, 70 mg/ml; penicillin, 100 units/ml; streptomycin sulfate, 100  $\mu$ g/ml; and insulin,  $2 \times 10^{-7}$  M. The perfusate was bubbled directly with CO<sub>2</sub>-O<sub>2</sub> 5:95 to maintain pH and oxygenation. During a 10-min nonrecirculated pre-perfusion, the Ca<sup>2+</sup>-free S77 medium contained 0.5 mM EDTA. Thereafter, the liver was perfused for approximately 15 min with recirculated medium containing 4 mM CaCl<sub>2</sub> and 0.03% (w/w) collagenase (Type CLS, Worthington Biochem. Corp., NJ). The softened liver was excised into 20 ml of LHSA medium (7) containing 0.02% collagenase, broken up with scissors, and the mixture was drawn twice into a wide-bore 25-ml pipette. The cell suspension was then shaken for 7 min in a total of 60 ml of LHSA medium containing 0.02% collagenase and was filtered through nylon mesh to remove undigested tissue. The filtrate was centrifuged for 3 min at 50 *g* and the pellet containing hepatocytes was washed once with LHSA medium and twice with the modified Tyrode's incubation buffer (see below).

The average cell yield from one liver was about  $3 \times 10^8$  cells. Initial viability judged by trypan blue staining was >95% and the respiration rate was in the range 14.2–19.6 nmol O<sub>2</sub> consumed/mg cell protein per min at 37°C, which is comparable with other reports (8).

For all hepatocyte suspension experiments, washed cells were resuspended in Tyrode's solution, pH 7.4, modified by the following additions: glucose 16.5 mM; HEPES 28 mM; bovine serum albumin fraction V 1%; essential amino acids in the proportions described by Hopgood, Clark, and Ballard, (9) 16 mM; glycine 1 mM; taurine 1 mM; penicillin 100 units/ml; and streptomycin sulfate 100  $\mu$ g/ml. The cells, density ca.  $5 \times 10^6$  cells/ml, were incubated at 37°C in 20-ml plastic vials (3 ml/vial) under a CO<sub>2</sub>-O<sub>2</sub> 5:95 atmosphere, with reciprocal shaking at 95 oscillations/min with an amplitude of 5 cm.

At zero time and after various periods of incubation, samples of cell suspension were removed for subsequent bile acid analysis either in total suspension or in the supernatant obtained by pelleting the hepatocytes at 50 *g* for 3 min. Cell protein was measured by the biuret reaction (10) and results were expressed as nmol bile acid present per mg cell protein.

#### Criteria of cell viability

At hourly intervals, cells were stained with trypan blue and the percentage of cells excluding the dye was determined. In addition, the release of lactate dehydrogenase from the cells during incubation was

followed, as was the stimulation of cellular respiration by 1 mM succinate (8), and the rate of cellular respiration was measured with a Clark oxygen electrode.

#### Bile acid analysis

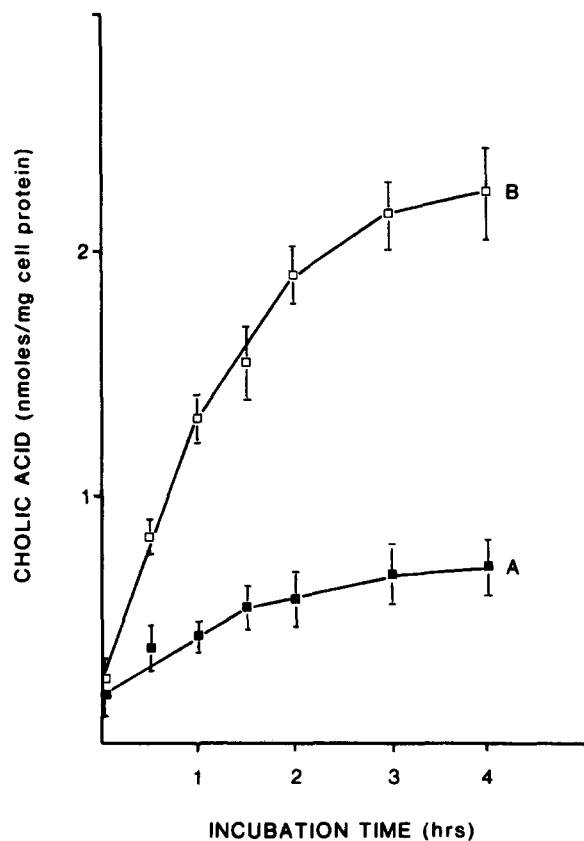
Bile acids were extracted from 2 ml of cell suspensions (ca. 20 mg protein) or cell supernatants by the addition of duplicate samples to 3 ml of ethanol containing 25 nmol of 7-ketodeoxycholic acid as an internal standard. After standing overnight, protein was removed by centrifugation at 2,000 *g* for 10 min, the supernatant was decanted, and the precipitate was washed with a further 2 ml of ethanol. Homogenization of cells prior to ethanol extraction did not change the amounts of bile acid recovered. The combined ethanol supernatants were evaporated to dryness on a rotary evaporator. Bile acids were then hydrolyzed with cholyglycine hydrolase (Sigma Chemical Co., St. Louis, MO) and extracted with ether and heptane as described by Ross, Pennington, and Bouchier (11).

Bile acids were converted to methyl esters with diazomethane. They were then trifluoroacetylated and quantitated using a Hewlett-Packard 5830 dual flame ionization gas chromatograph equipped with a 1.2 m  $\times$  2 mm i.d. glass column packed with 3% SP-2401 on Supelcoport 100–120 mesh (Supelco Inc., Bellefonte, PA). Separation was carried out at 245°C using nitrogen (30 ml/min) as carrier gas. Area ratios of cholic acid to internal standard were determined and samples were quantitated by comparison with taurocholate standards analyzed simultaneously.

The assay method was sensitive and precise. Recoveries of taurocholate or glycocholate (20 nmol) added at the beginning of the extraction procedure to a sample of liver cells were 85% and 86%, respectively, indicating nearly complete enzymatic hydrolysis of cholic acid conjugates. The coefficient of variation of replicate analyses ( $n = 4$ ) of cholic acid extracted from liver cells prior to incubation was 7.9%, and from liver cells incubated for 3 hr, 9.9%. The overall recovery of internal standard from the extraction procedure in 100 consecutive assays was  $89 \pm 15\%$  (mean  $\pm$  SD).

## RESULTS

The major bile acid present in rat bile collected prior to liver cell isolation was cholic acid. This bile acid accounted for an average of 55% of the total bile acids in untreated rats, and was increased significantly to 73% of the total bile acids in rats



**Fig. 1.** Synthesis of cholic acid during the incubation of rat liver cell suspensions. *A*, cells from normal rats; *B*, cells from rats fed a cholestyramine-containing diet. Each value is the mean of four experiments with the vertical bars indicating the SEM.

fed 4% (w/w) cholestyramine in the diet for 1 week. Chenodeoxycholic acid was also increased after cholestyramine feeding, from 9% to 15% of the total bile acids. These increases in the proportion of primary bile acids in rat bile suggest that cholestyramine was effectively binding bile acids in the intestine and largely preventing the return of secondary bile acids to the liver.

After isolation and washing with buffer at 37°C, liver cells still contained low levels of cholic acid (0.20

nmol/mg cell protein) and traces of other bile acids. Incubation of liver cell suspensions at 37°C produced a detectable increase only in cholic acid. The net increase in cholic acid was taken to represent new bile acid synthesis.

The time course of synthesis of cholic acid over a 4-hr incubation period is shown in **Fig. 1**. Rats fed a normal diet synthesized cholic acid at an initial rate of 0.25 nmol/hr per mg protein. By including cholestyramine in the diet for 1 week, the initial rate of cholic acid synthesis by liver cell suspensions was increased more than 4-fold to a value of 1.1 nmol/hr per mg protein. In both cases the rate of synthesis declined rapidly after 2 hr of incubation. This decline did not correspond to a sudden loss of cell viability at 2 hr as measured by trypan blue exclusion, leakage of lactate dehydrogenase, succinate stimulation of respiration, or respiration rate, as shown in **Table 1**. However, there was a slow decrease in cell viability over the entire incubation period. This decrease was minimal if the cells were stored on ice for 4 hr, and therefore probably represents mechanical damage to cells while shaking at 37°C.

The distribution of cholic acid between the cells and the incubation medium was also examined, and the amount of cholic acid secreted into the medium is shown in **Fig. 2**. In contrast to bile acid synthesis, the initial bile acid secretion rate was much lower than that observed after 2 hr of incubation. In rats fed a cholestyramine diet, the amount of cholic acid present extracellularly after 1 hr of incubation was 16% of the total amount present, while after 4 hr this value had increased to 69%. A similar pattern was observed in rats fed a normal diet. It is possible that the increased rate of accumulation of cholic acid in the medium after 2 hr of incubation is partly due to leakage from damaged cells, although only 29% of the total cellular lactate dehydrogenase was recovered in the supernatant after 4 hr.

**TABLE 1.** Cell viability during the incubation of rat liver cell suspensions

Test of Viability	Incubation Time (hr) <sup>a</sup>					
	0	1	2	3	4	4 at 0°C
Trypan blue exclusion (%)	96 ± 1	95 ± 1	94 ± 1	90 ± 1	77 ± 7	93 ± 1
Lactate dehydrogenase leakage (%)	5 ± 1	12 ± 1	17 ± 1	22 ± 2	29 ± 3	12 ± 1
Succinate-stimulated respiration ratio <sup>b</sup>	1.04 ± 0.01	1.08 ± 0.02	1.14 ± 0.03	1.12 ± 0.02	1.20 ± 0.09	1.15 ± 0.02
Respiration rate (%)	100	88 ± 4	80 ± 2	70 ± 4	58 ± 8	95 ± 2

<sup>a</sup> Each value represents the mean ± SEM of eight experiments.

<sup>b</sup> Respiration rate with 1 mM succinate divided by the respiration rate without succinate.

## DISCUSSION

The present work has established that by incubating rat liver cell suspensions at 37°C for up to 4 hr, synthesis of cholic acid, the major bile acid of rats, can be measured directly as a net increase in the bile acid present in cells plus cell medium. The initial rate of synthesis of cholic acid observed in normal Wistar rats was 0.25 nmol/mg protein per hr or 45 nmol/g liver per hr, which is comparable to a range of values obtained recently by different workers using a variety of *in vivo* methods, as summarized in Table 2. Yousef, Ho, and Jeejeebhoy (14) have also recently reported that isolated rat hepatocytes will synthesize bile acids, but these workers utilized more elaborate incubation conditions, including the presence of horse serum, an enriched medium, and controlled oxygen tension to maintain cellular functions for 24 hr.

Interruption of the enterohepatic cycle by cholestyramine feeding is known to increase the rate of bile acid synthesis in the rat by up to 8-fold (15). The finding that cholic acid synthesis in liver cells isolated from rats fed a cholestyramine diet was increased more than 4-fold over normal levels provides evidence that hepatocytes incubated in cell suspension retain adaptive changes in bile acid metabolism that have been produced *in vivo*. Our results then demonstrate that isolated hepatocytes incubated for short periods of 2–4 hr under simple conditions can be used to provide a measure of the rate of hepatic bile acid synthesis reflecting the *in vivo* rate. Further studies on the effects of sex hormones and other drugs on hepatic bile acid synthesis measured at the cellular level are therefore now possible.

Examination of the distribution of cholic acid between cells and medium using gas–liquid chromatography revealed that during the first 2 hr of incubation, when bile acid synthesis was maximal, only a small proportion of the bile acid was secreted into the medium. As the cells accumulated bile acid, secretion accelerated from an initial 0.05 nmol/mg protein per hr to 0.13 nmol/mg protein per hr. A similar pattern, but with higher secretion rates, was seen with cells from cholestyramine-treated rats. Factors influencing rates of secretion were not examined in any detail in this study.

While the rates for bile acid synthesis and secretion observed in this work are in reasonable agreement with previous *in vivo* measurements and with the results obtained with hepatocytes by Yousef et al. (14) using gas–liquid chromatography (see Table 2), these rates differ greatly from measurements of

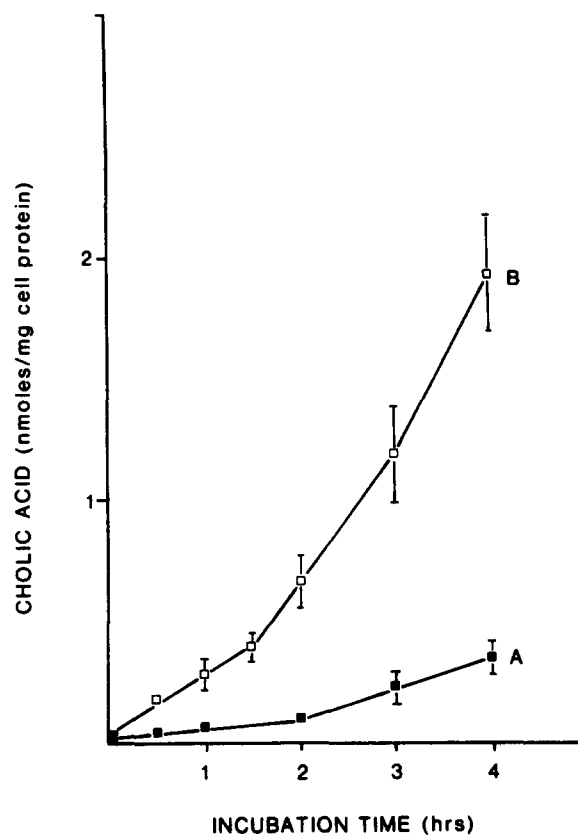


Fig. 2. Secretion of cholic acid during the incubation of isolated liver cells. A, cells from normal rats; B, cells from rats fed a cholestyramine-containing diet. Each value is the mean of 2–4 experiments, with the vertical bar indicating the SEM of four experiments.

rates of secretion in hepatocyte suspensions by Anwer, Kroker, and Hegner (16) and Gardner and Chenouda (17). Using the hydroxysteroid dehydrogenase enzymatic assay for bile acids, these workers


TABLE 2. Normal rates of hepatic bile acid synthesis in the rat as estimated by different techniques

Synthesis Rate	Rat Strain	Assay Technique	Reference
<i>nmol/hr/g liver</i>			
A. <i>In vivo</i>			
56	Wistar	Fecal output	12
106	Sprague-Dawley	Fecal output	12
86	Wistar	Fecal output	13
149	Wistar	Biliary drainage <sup>a</sup>	13
59	Sprague-Dawley	Biliary drainage <sup>a</sup>	5
205	Sprague-Dawley	[ <sup>14</sup> C]Cholesterol breakdown	5
B. <i>In vitro</i>			
21	Wistar	Isolated liver cells	14
45	Wistar	Isolated liver cells	Present study

<sup>a</sup> Assuming 5% of bile acid secreted is newly synthesized.



obtained rates of secretion, which they attributed to bile acid synthesis, of 23 nmol/g cells per min (equivalent to ca. 8 nmol/mg protein per hr) and 26–28 nmol/mg protein per hr, respectively. Gardner and Chenouda (17) also reported an apparent stimulation of bile acid secretion by succinate to as much as 916 nmol/mg protein per hr (ca. 160  $\mu$ mol/g liver per hr), a value that is 1000-fold higher than in vivo synthesis rates (Table 2). Furthermore they suggested that stimulation by succinate resulted from its utilization as respiratory substrate, but hepatocytes with intact plasma membranes are considered to be impermeable to succinate (8, 18). Indeed, in this study we have used the inability of succinate to stimulate respiration as an index of cell viability.

An explanation of these wide discrepancies in secretion rates is suggested by the finding that although the rate of cholic acid secretion measured in normal rat liver cell supernatants by gas-liquid chromatography in this study was 0.05 nmol/mg protein per hr, when the same samples were assayed by the enzymatic method much higher apparent secretion values of  $22 \pm 4.5$  (SEM) nmol/mg protein per hr were obtained, in agreement with Gardner and Chenouda (17). With the enzymatic assay, no difference was observed between cell supernatants from normal and cholestyramine-treated rats. Since the enzymatic assay measures only the generation of NADH and is not specific for bile acids, it is likely that the oxidation of another metabolite in the cell supernatant by a NAD-dependent dehydrogenase present in the crude bacterial extract, used as a source of hydroxysteroid dehydrogenase, interferes with the assay of bile acids by this method. Thus the conclusions of Anwer et al. (16) and Gardner and Chenouda (17) regarding rates of bile acid secretion and feedback inhibition of bile acid synthesis in isolated liver cells are based on incorrect values from the nonspecific enzymatic assay. Similarly, apparent stimulation of secretion by succinate, fumarate, and malate (17) may result from dehydrogenase activity unrelated to bile acid levels. Our results indicate that any studies of bile acid synthesis and secretion in isolated hepatocytes must use a specific and sensitive assay for bile acids, such as gas-liquid chromatography. 

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